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Abstract

A simple cell-free system for studying a priming factor involved in the repair of bleomycin-damaged DNA was established. The template-primer used for the repair DNA synthesis was prepared by treating the closed circular, superhelical form of pUC19 plasmid DNA with 2.2 microM bleomycin and 20 microM ferrous ions. Single-strand breaks were introduced into pUC19 DNA by the bleomycin treatment, and the DNA was consequently converted largely into the open circular form. A system for repair of this bleomycin-damaged DNA was constructed with a priming factor, DNA polymerase (DNA polymerase beta or Klenow fragment of DNA polymerase I), ATP, T4 DNA ligase and four deoxynucleoside triphosphates. After incubation, the conformation of the DNA was analyzed by agarose gel electrophoresis and electron microscopy. The open circular DNA was largely converted to the closed circular DNA, indicating that the single-strand breaks of DNA were repaired. When the priming factor was omitted, DNA repair did not occur. The present system seemed to be applicable to the study of priming factors involved in the repair of DNA with single-strand breaks caused not only by bleomycin but also by ionizing radiation or active oxygen.

KEYWORDS: priming factor, exonuclease, DNA repair, bleomycin, pUC19 DNA, agarosegel electrophoresis

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A Cell-Free System for Studying a Priming Factor Involved in Repair of Bleomycin-Damaged DNA

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A simple cell-free system for studying a priming factor involved in the repair of bleomycin-damaged DNA was established. The template-primer used for the repair DNA synthesis was prepared by treating the closed circular, superhelical form of pUC19 plasmid DNA with 2.2 μM bleomycin and 20 μM ferrous ions. Single-strand breaks were introduced into pUC19 DNA by the bleomycin treatment, and the DNA was consequently converted largely into the open circular form. A system for repair of this bleomycin-damaged DNA was constructed with a priming factor, DNA polymerase (DNA polymerase β or Klenow fragment of DNA polymerase I), ATP, T4 DNA ligase and four deoxynucleoside triphosphates. After incubation, the conformation of the DNA was analyzed by agarose gel electrophoresis and electron microscopy. The open circular DNA was largely converted to the closed circular DNA, indicating that the single-strand breaks of DNA were repaired. When the priming factor was omitted, DNA repair did not occur. The present system seemed to be applicable to the study of priming factors involved in the repair of DNA with single-strand breaks caused not only by bleomycin but also by ionizing radiation or active oxygen.

Key words : **priming factor, exonuclease, DNA repair, bleomycin, pUC19 DNA, agarose gel electrophoresis**

The typical DNA excision repair process in eukaryotic cells can be divided into four sequential steps: priming (recognition of DNA damage and incision-excision), repair DNA synthesis, repair patch ligation, and chromatin reorganization. The complexity of DNA repair mechanisms is thought to be due mainly to diversity in the priming step. Radiation causes various types of damage to DNA, and repair mechanisms are too complicated to be studied as a whole (1-3). Bleomycin, a radiomimetic anticancer drug, is known to cause two types of damage to DNA. The major type is a single-strand break resulting in 3'-phosphoglycolate and 5'-phosphate termini, and the other is the formation of alkali-labile sites (AP sites) (4-6). Both types of damage are known to be present also in DNA subjected to ionizing

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radiation (1, 7, 8). We have used bleomycin as a DNA damaging agent to establish a simple DNA repair system for analytical studies of DNA repair processes, and recently we established a DNA repair system using bleomycin-pretreated permeable HeLa cells (9). Further, we have partially purified a protein (priming) factor which exhibits exonucleolytic activity on bleomycin-damaged DNA and provides priming sites for DNA polymerases (10).

The present communication describes a simple method for detection of the priming factor involved in the repair of bleomycin-damaged DNA. The method was thought to be useful for detection and purification of not only priming factors but also other factors involved in repair of DNA with single-strand breaks.

Materials and Methods

Ribonucleotides (NTPs) and deoxyribonucleotides (dNTPs) were obtained from Seikagaku Kogyou Co., Ltd., Tokyo, Japan, and [^{3}H]dTTP was obtained from Amersham Japan Corp., Tokyo. Ferrous ammonium sulfate, Fe(II), was purchased from Katayama Chemicals, Osaka, Japan, ethidium bromide (EtdBr) from Nakarai Tesque Inc., Kyoto, Japan and agarose (Agarose standard low-m_r) from BioRad Labs, Richmond, CA, USA. Deferoxamine mesylate (Desferal®) was kindly provided by Japan Chiba Geigy Co., Ltd., Takarazuka, Japan. The other reagents used were obtained as described previously (10). Mouse ascites sarcoma (SR-C3H/He) cells were obtained and maintained as described previously (11).

Preparation of pUC19 DNA. Growth of HB-101 cells (a strain of *Escherichia coli*) transformed with pUC19 plasmid, amplification of the plasmid, harvesting and alkali lysis of the bacteria, and purification of the plasmid DNA were conducted by the large-scale isolation procedure described by Maniatis *et al.* (12). Closed-circular, superhelical DNA was purified by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient (12).

Preparation of bleomycin-Fe(II)-treated DNA. Closed-circular, superhelical plasmid DNA (0.3 mg) was incubated at 37℃ for 30 min with 2.2 μM bleomycin and 20 μM Fe(II) in 3 ml of Triton-buffer B (0.0175% Triton X-100, 0.25 M sucrose, 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol, pH 8.0) (13). After chilling to 0℃, 0.05 ml of 2.4 mM deferoxamine mesylate was added to the reaction mixture to cease the reaction by chelating Fe(II). The mixture was dialyzed at 4℃ against a solution (pH 8.0) containing 1 mM NaCl, 1 mM EDTA and 1 mM Tris-HCl to remove Fe(II), bleomycin and deferoxamine mesylate. NaCl (0.1 M) was added to the dialysate. DNA precipitated by adding 2 volumes of cold ethanol was dissolved in distilled water at 2.2 mg/ml.

Preparation of DNA polymerase β. SR-C3H/He ascites cells were permeabilized by treatment with buffer A containing 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol (pH 8.0), and stored at −20℃ until use. DNA polymerase β was extracted from permeable cells with 0.2 M potassium phosphate buffer at pH 7.5 in 6 mM 2-mercaptoethanol. The enzyme was partially purified by sequential chromatography with phosphocellulose, DEAE-cellulose and phosphocellulose (a second time), essentially according to the method of Chang (14, 15). The partially purified DNA polymerase β preparation thus obtained contained an exonuclease (priming factor) involved in the initiation of repair of bleomycin-damaged DNA (10). DNA polymerase β further purified by single-stranded DNA cellulose column chromatography was used in the present experiment.

Preparation of the priming factor (exonuclease). The priming factor was purified from the partially purified DNA polymerase β preparation by single-stranded DNA cellulose column chromatography as described previously (10).

Assay of template-primer activity of bleomycin-damaged DNA. The reaction mixture (60 μl in final volume) for assaying template-primer activity of bleomycin-damaged pUC19 DNA consisted of 40 μl Triton-buffer B containing 6 μg closed-circular pUC19 DNA, 20 μM Fe(II), 5 μl of priming factor solution, various amounts of bleomycin and 0.04 unit Klenow fragment of DNA polymerase I (Klenow polymerase), and 20 μl of a substrate mixture for DNA synthesis (100 mM Tris-HCl, 7.5 mM $MgCl_2$,

240 mM NaCl, 150 μM dATP, 30 μM dCTP, 150 μM dGTP and 7.5 μM [^{3}H]dTTP at 5 Ci/mmol, pH 8.0 at 25℃). DNA damage caused by bleomycin proceeded gradually at 0℃. Therefore, incubation for assay of template-primer activity was started within 1 h after the addition of bleomycin. DNA synthesis was conducted at 37℃ for 30 min, and then stopped by chilling in an ice-water bath. Fifty μl of each reaction mixture was pipetted onto a glassfiber disc (Whatman GF/C) numbered with India ink. The discs were batch washed in 5% trichloroacetic acid, 95% ethanol and ether as described previously (10, 14). The radioactivity was measured as described previously (11).

Repair of pUC19 DNA with bleomycin-induced single-strand breaks. A system for repairing bleomycin-Fe(Ⅱ)-treated pUC19 DNA was constructed. The reaction mixture (adjusted to a final volume of 30 μl with Triton-buffer B) consisted of 3 μg bleomycin-Fe(Ⅱ)-treated pUC19 DNA, 2.5-5 μl of priming factor solution, 5 μl DNA polymerase β (or 0.02 unit Klenow polymerase), 2.5 mM ATP, 20-40 units T4 DNA ligase and 10 μl of a substrate mixture for DNA synthesis. The compositions and concentrations of the substrate mixture were the same as those described above except that dTTP replaced [^{3}H]dTTP and that NaCl was omitted. The mixture was incubated at 37℃ for 30-180 min, and then chilled to 0℃.

Agarose gel electrophoresis. Agarose gels were prepared at 0.8% in the TBE buffer (0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA, pH 8.0) supplemented with EtdBr at 0.5 μg/ml unless otherwise indicated (12). The electrophoresis buffer was TBE buffer supplemented with EtdBr at 0.5 μg/ml. Five volumes of each sample were mixed with a volume of a 6-fold-concentrated gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in H_2O), and a portion of the mixture was loaded into a slot of a submerged agarose gel. Electrophoresis was conducted at 50 volts for 90 to 120 min using a Mini Gel Electrophoresis System (Mupid 2) of Cosmo Bio Co., Ltd., Tokyo.

Electron microscopic examination. DNA was mounted on a carbon-covered, collodion-coated grid by a modified procedure of the protein monolayer technique of Kleinschmidt *et al.* (16) and Mayor and Jordan (17). The spreading solution contained about 5 μg/ml DNA in 1 M ammonium acetate, 0.01% cytochrome *c* and 0.5% formaldehyde. The hypophase consisted of 0.3 M ammonium acetate and 0.5% formaldehyde. A small drop of the spreading sample was placed on the surface of a large drop of the hypophase on a parafilm sheet. Immediately after spreading, DNA in the cytochrome monolayer was picked up on the collodion-coated grid. The excess liquid adhering to the grid was blotted, and the grid was dehydrated by touching it to the surface of absolute ethanol and draining dry on filter paper. The grid was then rotary shadowed with platinum-palladium (8:2), and examined with an HU-11C electron microscope (Hitachi Ltd., Tokyo).

Results and Discussion

Bleomycin-induced DNA damage and template-primer activity of the damaged DNA. Damage to pUC19 DNA due to bleomycin treatment increased in a dose-dependent fashion at drug concentrations over 0.22 μM (Fig. 1). Undamaged, superhelical DNA was not detected after treatment with bleomycin at concentrations of 5.5 μM or higher (Fig. 1). DNA fragments shorter than the linear pUC19 DNA appeared and increased in a dose-dependent fashion at bleomycin concentrations over 2.2 μM. Template-primer activity of the pUC19 DNA treated with bleomycin increased in a damage-dependent fashion at bleomycin concentrations between 0.55 and 11 μM (Fig. 2). The initial preparation of DNA (largely superhelical DNA) showed low template-primer activity, about one-tenth of the maximum activity attained in the presence of bleomycin. Bleomycin-damage-dependent DNA synthesis was hardly detected without the priming factor (exonuclease). In the following experiments, pUC19 DNA treated with 2.2 μM bleomycin was used as the template-primer for studying repair of bleomycin-induced, single-strand breaks, because single-strand breaks were introduced most effectively at this bleomycin concen-

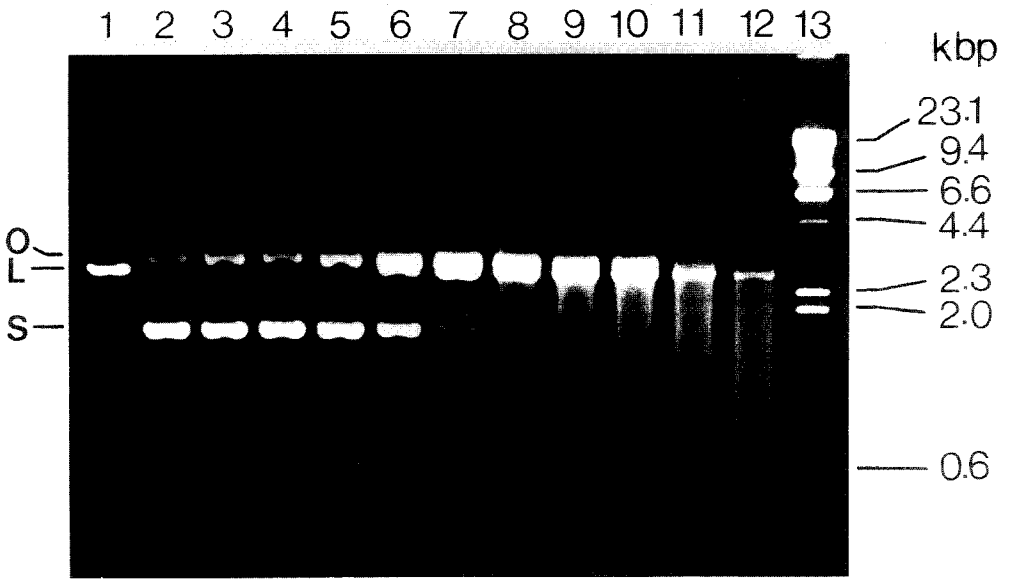

Fig. 1 Effects of varying the concentration of bleomycin on DNA damage. The reaction mixture for measuring bleomycin-induced DNA damage was the same compositions and concentrations as that for assaying template-primer activity of bleomycin-damaged pUC19 DNA as described in Materials and Methods except that the total volume was reduced to 30 μl and that dTTP replaced [^{3}H]dTTP. The mixture was incubated at 37℃ for 30 min, and then chilled to 0℃. Immediately after incubation, DNA was analyzed by agarose gel electrophoresis. The amount of DNA loaded into each slot of lanes 3 to 12 was 0.42 μg. Lane 1, EcoR$_1$-treated pUC19 DNA (a marker of linear pUC19 DNA); lane 2, the original preparation of pUC19 DNA; lanes 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12, pUC19 DNA treated with bleomycin at 0, 0.22, 0.55, 1.1, 2.2, 5.5, 11, 22, 55 and 110 μM, respectively; lane 13, λ DNA digested with Hind III. Abbreviations: O, open circular DNA; L, linear DNA; S, closed circular, superhelical DNA; kbp, kilobase pairs.

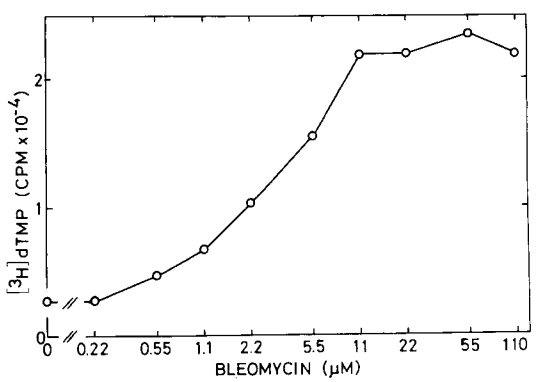

Fig. 2 Effects of the concentration of bleomycin on template-primer activity of pUC19 DNA. The reaction mixture for assaying template-primer activity of bleomycin-damaged DNA in the presence of the priming factor (exonuclease) and the assay procedure were as described in Materials and Methods. Data are expressed as cpm of [^{3}H]dTMP incorporated in 30 min into DNA in 0.06 ml of the reaction mixture.

tration.

Repair of bleomycin-induced, single-strand breaks. A repair system for bleomycin-Fe (II)-treated pUC19 DNA was constructed using the priming factor, DNA polymerase β and T4 DNA ligase, as described in Materials and Methods. When the repair reaction was conducted with the complete assay mixture and the sample was analyzed by agarose gel electrophoresis, a thick new band (R band) appeared, and the band of open circular DNA (O band) decreased markedly (Fig. 3, lane 3). The DNA forming the R band was considered to be repaired DNA. When the priming factor, T4 DNA ligase or DNA polymerase β was omitted from the assay mixture, the R band was not detected (Fig. 3, lane 4, 6 or 7). The appearance

of a faint R band in the absence of ATP (lane 5) might be due to a trace amount of ATP contaminating the deoxyribonucleoside triphosphates in the assay mixture (18). Essentially the same result (DNA repair) was obtained when the partially purified DNA polymerase β fraction containing the priming factor was used in place of the purified priming factor preparation and Klenow polymerase was used in place of DNA polymerase β (data not shown).

Conformational analyses of the repaired DNA. The sample incubated for repair DNA

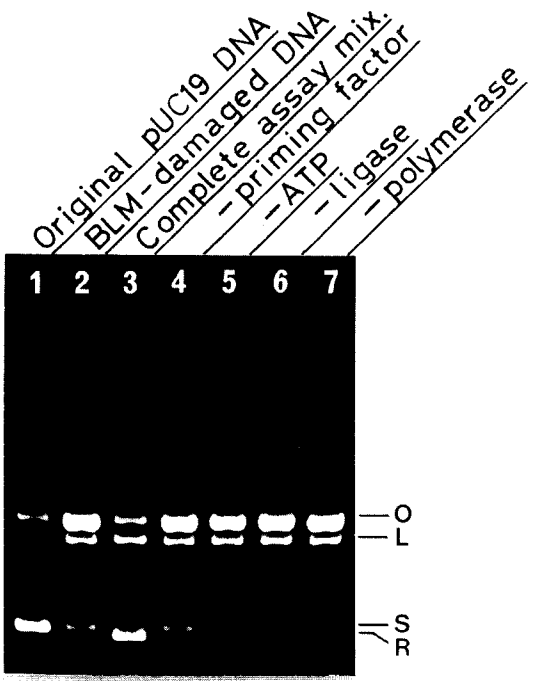

Fig. 3 Analyses by agarose gel electrophoresis of a DNA repair system. The complete assay mixture for DNA repair contained bleomycin-Fe(II)-treated DNA, the priming factor, DNA polymerase β, ATP and T4 DNA ligase. After incubation at 37℃ for 60 min, agarose gel electrophoresis was performed. The amount of DNA loaded into each slot was 0.25 μg. Lane 1, the original preparation of pUC19 DNA; lane 2, the bleomycin-Fe (II)-treated pUC19 DNA (BLM-damaged DNA); lane 3, the bleomycin-Fe(II)-treated pUC19 DNA incubated in the complete assay mixture. The priming factor was omitted from the complete assay mixture in lane 4; ATP, in lane 5; T4 DNA ligase, in lane 6, and DNA polymerase β, in lane 7. Abbreviations: O, open circular DNA; L, linear DNA; S, closed circular, superhelical DNA; R, repaired, closed circular DNA.

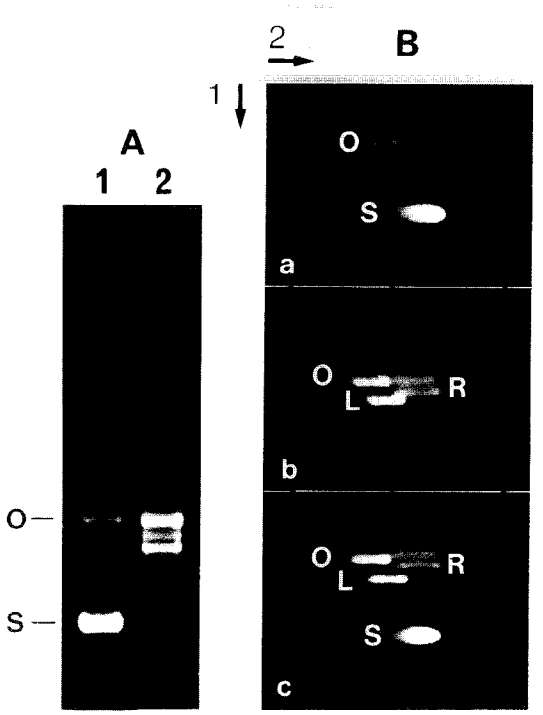

Fig. 4 Two dimensional agarose gel electrophoretic analyses of pUC19 DNA after incubation for repair. Fig. 4A. Gel electrophoresis was conducted with 1% agarose gel prepared without EtdBr, and without EtdBr in the electrophoresis buffer. The amount of DNA loaded into each slot was 0.33 μg. After the electrophoresis, DNA was stained with EtdBr by gently shaking the gel in TBE buffer supplemented with EtdBr at 0.5 μg/ml. Lane 1, the original preparation of pUC19 DNA; lane 2, the same preparation of bleomycin-Fe(II)-treated pUC19 DNA incubated for DNA repair in the complete assay mixture described in the legend to Fig. 3. Fig. 4B. Gel electrophoresis was conducted at 50 volts for 90 min without EtdBr in the direction of arrow 1, as described above (Fig. 4A). The gel was shaken gently for 90 min in TBE buffer supplemented with EtdBr at 0.02 μg/ml, and then electrophoresed at 50 volts for 90 min in the direction of arrow 2 in TBE buffer supplemented with EtdBr at 0.02 μg/ml. After electrophoresis, DNA was stained with EtdBr by shaking gently the gel in TBE buffer supplemented with EtdBr at 0.5 μg/ml. a, the original preparation of pUC19 DNA; b, the same preparation as that shown in Fig. 4A, lane 2; c, the mixture of the above two preparations. The abbreviations used are the same as in the legend to Fig. 3.

synthesis in the complete assay mixture contained DNA forming the R band, open circular DNA and linear DNA (Fig. 3, lane 3). When the same sample was electrophoresed in the absence of EtdBr, bands forming a ladder were observed around the bands of open circular DNA and linear DNA (Fig. 4A, lane 2). Since electrophoretic mobility is influenced by the number of superhelical turns, and heterogeneity of the number of superhelical turns occurs in repaired DNA as a consequence of thermal fluctuations in the DNA helix at the time when the last phosphodiester bond is closed by DNA ligase (19), the DNA bands forming the ladder are considered to be repaired DNA molecules which are in relaxed circular form with a few topological isomers. Thus, the DNA forming the ladder without EtdBr was thought to be the DNA forming the R band in the presence of EtdBr at 0.5 μg/ml. When the same sample was first electrophoresed in an agarose gel without EtdBr (1st dimension) and then in the presence of EtdBr (2nd dimension), at least three of the bands forming the ladder moved faster than the open circular or linear DNA, indicating that these were repaired, closed circular DNA with different superhelical turns (Fig. 4B).

The conformational changes of pUC19

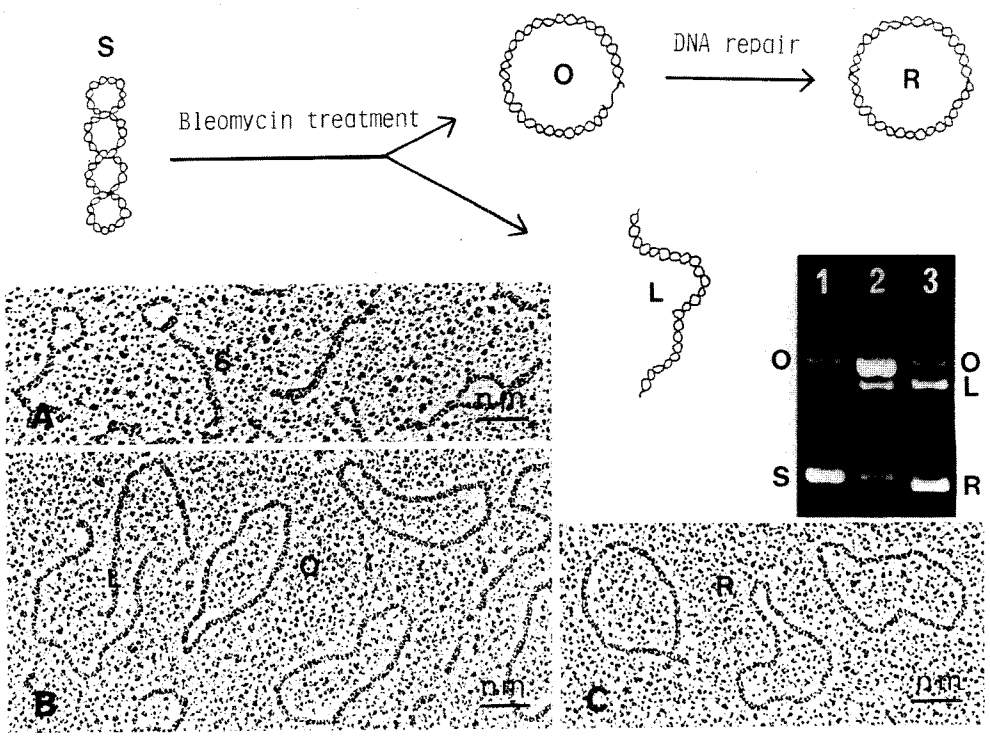

Fig. 5 Conformational changes occurring in pUC19 DNA during damage by bleomycin treatment and repair in the complete assay mixture. The conformations observed in electron micrographs (A, B, C) and in an agarose gel electrophoretogram (right) are represented schematically (top). Electromicrograms: A, the original preparation of pUC19 DNA; B, the bleomycin-Fe(II)-treated DNA: C, the bleomycin-damaged DNA incubated for DNA repair in the complete assay mixture. Agarose gel electrophoretogram: Lane 1, the original preparation of pUC19 DNA; lane 2, the bleomycin-Fe(II)-treated pUC19 DNA; lane 3, the bleomycin-Fe(II)-treated DNA incubated for DNA repair in the complete assay mixture. The abbreviations used are the same as described in the legend to Fig. 3.

DNA were also studied electron-microscopically. Electron micrographs of the original preparation showed mostly closed-circular, superhelical DNA (Fig. 5A). The electron micrographs of the bleomycin-Fe(II)-treated DNA and the DNA incubated in the complete assay mixture, both showed apparently open circular DNA and linear DNA (Fig. 5B and 5C). In consideration of that electron microscopic discrimination between nicked open circular DNA (O) and the relaxed closed circular DNA (R) is difficult, the results of the electron microscopic examination appear to agree with those obtained by agarose gel electrophoresis. Fig. 5 also shows a schematic representation of the conformational changes of pUC19 DNA in the process of bleomycin-induced DNA damage and repair of the damaged DNA in the complete assay mixture.

The results of the present study suggest that utilization of bleomycin-damaged pUC19 DNA as a template-primer and agarose gel electrophoresis for detection of repaired DNA would be useful for the detection, purification and characterization of not only priming factors but also other factors involved in the repair of DNA with single-strand breaks. Conformational analysis of circular DNA by agarose gel electrophoresis is simple, highly sensitive and does not require radioactive isotopes.

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